

Enhanced rate of intramolecular electron transfer in an engineered purple Cu_A azurin

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ABSTRACT The recent expression of an azurin mutant where the blue type 1 copper site is replaced by the purple Cu_A site of *Paracoccus denitrificans* cytochrome *c* oxidase has yielded an optimal system for examining the unique electron mediation properties of the binuclear Cu_A center, because both type 1 and Cu_A centers are placed in the same location in the protein while all other structural elements remain the same. Long-range electron transfer is induced between the disulfide radical anion, produced pulse radiolytically, and the oxidized binuclear Cu_A center in the purple azurin mutant. The rate constant of this intramolecular process, $k_{ET} = 650 \pm 60 \text{ s}^{-1}$ at 298 K and pH 5.1, is almost 3-fold faster than for the same process in the wild-type single blue copper azurin from *Pseudomonas aeruginosa* ($250 \pm 20 \text{ s}^{-1}$), in spite of a smaller driving force (0.69 eV for purple Cu_A azurin vs. 0.76 eV for blue copper azurin). The reorganization energy of the Cu_A center is calculated to be 0.4 eV, which is only 50% of that found for the wild-type azurin. These results represent a direct comparison of electron transfer properties of the blue and purple Cu_A sites in the same protein framework and provide support for the notion that the binuclear purple Cu_A center is a more efficient electron transfer agent than the blue single copper center because reactivity of the former involves a lower reorganization energy.

The Cu_A centers (1) serve as the electron uptake site in the terminal respiratory enzyme cytochrome *c* oxidase (2) and also as a redox center in nitrous oxide reductase (N₂OR) (3). A combination of x-ray structural characterization (4–7) and spectroscopic studies (for example, see refs. 8–17) on native enzymes, water-soluble fragments containing the Cu_A center (18–20), engineered Cu_A centers (21–23), and inorganic model compounds (24, 25) has established Cu_A as a mixed valence [Cu(1.5) – Cu(1.5)] (*S* = 1/2) center with two copper ions in a Cu₂S₂ diamond core, and these studies have provided a firm basis for understanding the structure and function of this class of biological copper centers. An immediate question that this unusual structure raised was what functional advantage has led to its selection, in particular compared with the type 1 (T1) blue copper centers. At least two distinct, though not mutually exclusive, rationales have been brought up so far. One is that the delocalized mixed-valence structure of the Cu_A site would facilitate the unidirectional long-range electron transfer (ET) to the cytochrome *a* site of the enzyme (11, 26). The other suggested that the Cu_A structure would yield a lower reorganization energy, as the metal-ligand bond length changes upon ET would amount to only half of those occurring in a mononuclear site (11, 14, 27).

To address the above question, ET studies on both the blue copper (28–31) and the purple Cu_A proteins (26, 32–36) have been carried out. An ideal system to directly answer the above

question will be a well-characterized protein where either the blue copper or the purple Cu_A center is placed at the same location and ET is known to occur through the same protein framework. In this way many parameters affecting the direct comparison of ET efficiency of the two copper centers (such as different protein sequences and pathways) can be minimized and the difference between the two distinct copper centers can be highlighted.

As part of our ongoing efforts to understand long-range ET processes in proteins (30) and to redesign metalloproteins (37), we have used the blue copper protein azurin as one of our model systems. Azurin consists of rigid β -pleated sheets and contains two potential redox centers: the T1 blue copper ion coordinated directly to amino acid residues, and a disulfide bridge (RSSR) present at the opposite end of the molecule, separated by a direct distance of 2.65 nm. Intramolecular ET between these sites was investigated in a large number of wild-type and single site-directed mutant azurins (38, 39), and the effect of specific changes in the protein structure on electronic couplings, reorganization energies, and the nature of the medium separating donor and acceptor were examined (30). We have succeeded in engineering an azurin variant (called purple Cu_A azurin hereafter) where the blue copper site has been replaced by the purple Cu_A center (23). Comprehensive spectroscopic characterization of the engineered azurin from *Pseudomonas aeruginosa* has demonstrated the striking similarity between the purple Cu_A azurin and the native purple Cu_A centers (11, 23, 40–43). The three-dimensional structure of the engineered purple Cu_A azurin at 1.65 Å (see supplemental data on the PNAS web site, www.pnas.org) shows close overall structural similarity between the native blue copper azurin and the engineered purple Cu_A azurin with an rms deviation of 1.07 Å for the α -carbon backbone when the ligand loop (corresponding to Cys-112 to Met-121 in the native blue copper azurin) is not considered in the calculation. Therefore, placing either a blue copper or a purple Cu_A center into the same protein framework permits a critical comparison of the efficiency of the two centers as electron mediators. The results of kinetic studies presented here suggest that the Cu_A center is a relatively more efficient ET agent because of its lower reorganization energy.

MATERIALS AND METHODS

Sample Preparation. The purple Cu_A azurin was prepared and purified to homogeneity as described (23, 43). [Ru(NH₃)₅Py](ClO₄)₂ (Py = pyridine) was synthesized according to the procedure outlined by Cummins and Gray (44).

Redox Potential Measurements on Ru[(NH₃)₅Py](ClO₄)₂. Redox potential measurements were made by using a Bioanalytical Systems (West Lafayette, IN) CV-50W potentiostat with a platinum working electrode, platinum auxiliary elec-

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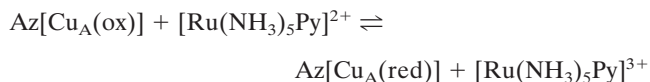
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Abbreviations: ET, electron transfer; T1, type 1; Py, pyridine; RSSR, the disulfide bridge between Cys-3 and Cys-26 in azurin.

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trode, and a Ag/AgCl reference electrode, all supplied by Bioanalytical Systems.

Redox Titrations. Titrations were performed by using 0.3–0.4 mM purple Cu_A azurin in 50 mM ammonium acetate, pH 5.1 and [Ru(NH₃)₅Py](ClO₄)₂ under a flow of Ar. The reduction of purple Cu_A azurin was monitored by Cu_A absorption at 774 nm where the [Ru(NH₃)₅Py]³⁺ and [Ru(NH₃)₅Py]²⁺ have no contribution to absorption. Each titration consisted of 8–9 aliquots of freshly prepared [Ru(NH₃)₅Py](ClO₄)₂ solution under Ar. Corrections were made for dilution. For the reaction,



the equilibrium constants were obtained according to Eq. 1 (45),

$$K = \left[\frac{A_{774}^\circ - A_{774}}{A_{774}} \right] \left[\frac{\epsilon_{774}[\text{Ru}]_t}{A_{774}^\circ - A_{774}} - 1 \right]^{-1}, \quad [1]$$

where A_{774} , A_{774}° , ϵ_{774} , and $[\text{Ru}]_t$ are absorbance of purple Cu_A azurin, absorbance of fully oxidized purple Cu_A azurin, its extinction coefficient at 774 nm, and total concentration of ruthenium ions.

Kinetic Measurements. The pulse radiolysis system using the Varian V-7715 linear accelerator at the Hebrew University in Jerusalem was used for carrying out the kinetic experiments (46). By using 5-MeV accelerated electrons and pulse lengths ranging from 0.1 to 1.5 μs , we produced 0.6–10 μM of CO₂^{•−} radical ions. All optical measurements were carried out anaerobically under purified N₂O in a 4 × 2 × 1 cm Spectrosil cuvette. Three light passes were used, which result in an overall optical path length of 12.3 cm. A 150-W xenon lamp produced the analyzing light beam, and an appropriate optical filter with cut-off at 385 nm was used to avoid photochemistry and light scattering. The data acquisition system consisted of a Tektronix 390 A/D digitizer connected to a personal computer. The temperature of the reaction solutions was controlled by a thermostating system and continuously monitored by a thermocouple attached to the cuvette (46). Practically all reactions were performed under pseudo-first order conditions, with typically a 10-fold excess of oxidized protein over reductant. The concentration of oxidized Cu_A was monitored at 485 and 510 nm, while formation and decay of the RSSR^{•−} radical was followed at 410 nm ($\epsilon_{410} \cong 10,000 \text{ M}^{-1}\text{cm}^{-1}$) (47). Kinetic runs at each temperature were repeated at least three times.

Aqueous solutions, 0.1 M in sodium formate (pH 5.1), were deaerated and saturated with N₂O in glass syringes. Afterward the concentrated protein stock solution was added and N₂O bubbling was continued for another 5 min. The solutions then were transferred into the pulse radiolysis cuvette under anaerobic conditions.

RESULTS

Fig. 1 shows a typical example of the time-dependent reduction of oxidized purple Cu_A azurin monitored at 485 nm and RSSR^{•−} reoxidation at 410 nm. The pulse radiolytically produced CO₂^{•−} radicals led to reduction of purple azurin ($k = 8 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ at 25°C, pH 5.1). The concentration of CO₂^{•−} radicals produced was controlled so that less than 5% of the protein electron acceptor sites were reduced during a single pulse. Hence the probability for any azurin molecule being reduced by more than one electron during one pulse is quite low. A similar fast reduction can be monitored at 410 nm where the produced RSSR^{•−} radical anion absorbs. These fast (diffusion controlled) bimolecular reactions (not shown) are followed by a slower process (Fig. 1), which is the result of ET from the disulfide radical ion (Fig. 1B) to the oxidized Cu_A

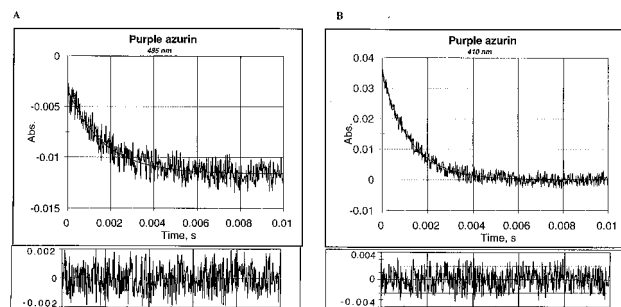


FIG. 1. Time-resolved absorbance changes of a 4.3 μM purple Cu_A azurin solution after a pulse of accelerated electrons at (A) 485 nm, the intramolecular reduction of mixed valence [Cu(1.5)-Cu(1.5)] Cu_A azurin and (B) 410 nm, the decay of the RSSR^{•−} radical. Conditions were: N₂O saturated aqueous solution, 100 mM sodium formate, pH 5.1; temperature, 25.0°C; pulse width, 0.4 μs . (Lower) The residuals of the fitting procedure.

center (Fig. 1A). The observed rate constants of these processes were the same ($k_{\text{ET}} = 650 \pm 60 \text{ s}^{-1}$, pH 5.1, 25°C) and were independent of both protein and radical concentrations, demonstrating that it proceeds intramolecularly. The temperature dependence of this process was examined for both blue copper (i.e., wild-type *P. aeruginosa* azurin) and the engineered purple Cu_A azurin ranging from 0.5°C to 40°C for the former and 3.5°C to 42.8°C for the latter. The first-order rate constant, k_{ET} , can be written as:

$$k_{\text{ET}} = A' \cdot \exp \left\{ -\frac{E_a}{R \cdot T} \right\}. \quad [2]$$

Accordingly, a plot of $\ln(k_{\text{ET}})$ vs. $1/T$ for both proteins is shown in Fig. 2. From these data the activation enthalpy was determined to be: $\Delta H^\ddagger = E_a - RT = 33.7 \pm 3.1 \text{ kJ} \cdot \text{mol}^{-1}$ for purple Cu_A azurin, and $\Delta H^\ddagger = 36.5 \pm 3.0 \text{ kJ} \cdot \text{mol}^{-1}$ for the native blue copper azurin. The activation entropy ΔS^\ddagger , including the contribution from the electronic factor $\kappa(r)\nu$ for a nonadiabatic ET (compare with Eq. 5 below) can be calculated from:

$$A' = \frac{k_B \cdot T \cdot e}{h} \cdot \exp \left\{ \frac{\Delta S^\ddagger}{R} \right\} \quad [3]$$

and was found to be $\Delta S^\ddagger = -78 \pm 5 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ for purple Cu_A azurin, and $\Delta S^\ddagger = -74 \pm 6 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ for the native blue copper azurin at pH 5.1 and 298 K.

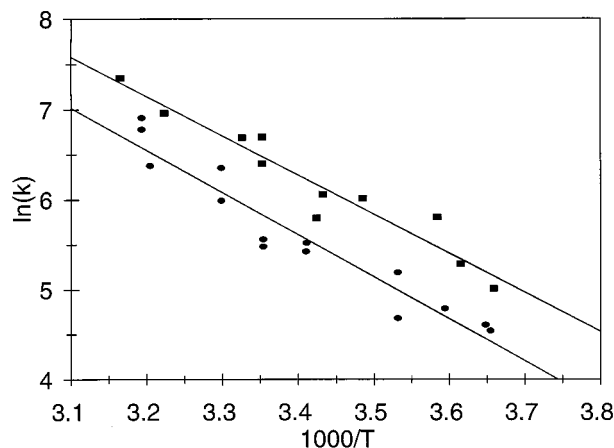
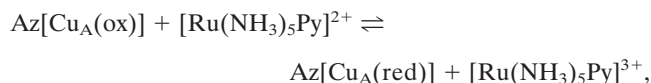


FIG. 2. Temperature dependence of intramolecular RSSR^{•−} to Cu ET in purple Cu_A (■) and wild-type blue copper (●) azurin from *P. aeruginosa* shown as a plot of $\ln(k_{\text{ET}})$ vs. $1/T$. The activation parameters were determined from this plot according to Eqs. 2 and 3.

Redox titrations of the purple Cu_A azurin were carried out at pH 5.1 and 298 K by using [Ru(NH₃)₅Py]²⁺. For the reaction



the equilibrium constants were found to vary slightly with different concentrations of [Ru(NH₃)₅Py]²⁺ between 0.1 and 2.0 mM. The final equilibrium constant ($K = 0.48$) was obtained by extrapolation to zero concentration of [Ru(NH₃)₅Py]²⁺. From the above K and the measured $E^\circ = 302$ mV for [Ru(NH₃)₅Py]^{3+/2+} couple in 50 mM ammonium acetate (pH 5.1), a reduction potential of 283 mV is obtained for the purple Cu_A azurin. This value is lower than the potential of the T1 center in *P. aeruginosa* azurin (350 mV at pH 5) (48) and higher than the value of 240 mV reported for the Cu_A site in cytochrome *c* oxidase fragment (49). Hence, the driving force for the internal ET in purple azurin is lower than that operating in the wild-type protein. The reversibility of the above reaction was ascertained by using Ce(IV) as oxidant, which led to practically full reoxidation of the Cu_A center.

DISCUSSION

As the only change introduced into wild-type azurin is the replacement of the T1 site by that of Cu_A, comparing long-range ET between RSSR⁻ and the respective copper centers in wild-type azurin and the purple azurin is expected to yield a better understanding of the ET properties of the Cu_A site. The intramolecular ET has been studied earlier in a large number of wild-type and site-directed mutant azurins (38, 39). The pH dependence of intramolecular ET exhibited a dramatic increase in the rate with decreasing pH. Thus, at pH 5.1 the rate constant k_{ET} becomes $250 \pm 20 \text{ s}^{-1}$ (50). The rate constant for the intramolecular ET in purple azurin is almost 3-fold larger than that of blue azurin at low pH, $k_{\text{ET}} = 650 \pm 60 \text{ s}^{-1}$.

According to the semiclassical Marcus theory (51) the ET rate constant depends on the electronic coupling constant, H_{DA} between electron donor (D) and acceptor (A), on the reorganization energy, λ and on the driving force, ΔG^0 :

$$k_{\text{ET}} = \kappa(r) \cdot \nu \cdot \exp \left\{ -\frac{(\Delta G^0 + \lambda)^2}{4 \cdot \lambda \cdot R \cdot T} \right\}. \quad [4]$$

In the nonadiabatic regime, ($\kappa \ll 1$),

$$\kappa(r) \cdot \nu = \frac{2 \cdot \pi}{\hbar} \cdot \frac{H_{\text{DA}}^2}{(4 \cdot \pi \cdot \lambda \cdot R \cdot T)^{1/2}}, \quad [5]$$

where the electronic coupling decreases exponentially with the distance:

$$H_{\text{DA}} = H_{\text{DA}}^0 \cdot \exp \left\{ -\frac{\beta}{2} \cdot (r - r^0) \right\}. \quad [6]$$

The question is then what causes the enhancement of the intramolecular ET rate in purple Cu_A azurin compared with the native blue copper protein.

The reduction potential for wild-type *P. aeruginosa* azurin at pH 5 is 350 mV (48). With a potential of -410 mV for the RSSR/RSSR⁻ couple (47), the driving force for intramolecular RSSR⁻ to Cu(II), $-\Delta G^0$ is $73.3 \text{ kJ} \cdot \text{mol}^{-1}$ (0.76 eV). The standard potential of the binuclear Cu_A center in the engineered azurin was determined to be 283 mV at pH 5.1. The driving force $-\Delta G^0 = 66.9 \text{ kJ} \cdot \text{mol}^{-1}$ (0.69 eV) is now calculated. In spite of this smaller driving force in the latter, the rate of intramolecular ET in the purple Cu_A protein is nevertheless faster than in the blue copper protein.

An ET pathway in *P. aeruginosa* azurin from Cys-3 (i.e., part of the disulfide bond) to Cys-112 (a copper ligand in both blue and purple azurin) has been proposed (30). It links Cys-3 via a hydrogen bond to Thr-30 and further from Val-31 to Trp-48, by a 0.40 nm through-space jump. Then, Val-49 and Phe-111 are connected through another H-bond, followed by a backbone connection to the Cys-112 copper ligand. The close overall structural similarity between the native blue copper azurin and the engineered purple Cu_A azurin has been demonstrated recently by x-ray crystallography (see Fig. 3, which is published as supplemental data on the PNAS web site, www.pnas.org). An rms deviation taken over the residues separating electron donor and acceptor was calculated to be 0.24 Å (see Fig. 4, which is published as supplemental data on the PNAS web site, www.pnas.org). The same number of covalent bonds, the same two H-bonds and the through-space jump all are found in the purple Cu_A azurin structure, too. The hydrogen bonds are slightly longer (≈ 0.1 Å) in the mutant protein while the van der Waals contact distances are essentially identical (3.83 Å in wild-type azurin vs. 3.79 Å in the purple Cu_A azurin). Therefore, the same pathway is most probably also operative in the engineered purple azurin. Finally, it should be noted that the Cu-S covalency of the blue copper center is $\approx 38\%$, but it is slightly lower in the Cu_A center, where the total Cu-S covalency has been calculated to be 26% (11, 15). Thus, there is no structural indication for an improved overlap of donor and acceptor wave functions.

We previously have determined the total reorganization energy (including both electron donor [RSSR⁻] and acceptor [Cu(II)]) of wild-type *P. aeruginosa* azurin to be 1.0 eV (28). By using k_{ET} of 650 s^{-1} for the intramolecular ET in purple azurin we calculate a $\lambda_{\text{TOT}} = 0.81 \text{ eV}$. Here λ_{TOT} is related to the reorganization energies of the individual redox centers (51):

$$\lambda_{\text{TOT}} = \frac{\lambda_{11}}{2} + \frac{\lambda_{22}}{2}. \quad [7]$$

Di Bilio *et al.* (31) recently have analyzed the intramolecular long-range ET in Ru(II) modified azurin and calculated reorganization energies of both electron donor and acceptor centers from the temperature dependence of the observed rate constants (31). For the blue copper center $\lambda = 0.82 \text{ eV}$ was reported. Now, if λ_{11} in Eq. 7 denotes reorganization of the copper center whereas λ_{22} is that of the disulfide group, we can calculate the latter from the previously determined values of $\lambda_{\text{TOT}} = 1.0 \text{ eV}$ and $\lambda_{11} = 0.82 \text{ eV}$ in wild-type *P. aeruginosa* azurin to be $\lambda_{22} = 1.2 \text{ eV}$. Assuming that this energy is unaffected by the substitution of the blue copper with the purple Cu_A center and by using the presently determined $\lambda_{\text{TOT}} = 0.81 \text{ eV}$ for purple azurin we now calculate $\lambda_{11} = 0.4 \text{ eV}$ for reorganization of the Cu_A center. Hence the reorganization energy of this site is only 50% that of the blue copper site. This finding constitutes strong experimental support for the notion that Cu_A is indeed a redox center with a more facile ET ability.[¶]

ET from Cu_A to heme *a* in cytochrome *c* oxidase is remarkably faster ($k_{\text{ET}} \approx 10^4 \text{ s}^{-1}$) than what we observe here in the purple Cu_A azurin, particularly considering the much smaller driving force (0.05 eV) (35). However, an ET pathway has been identified by Ramirez *et al.* (26, 36), which links the two redox centers in cytochrome *c* oxidase. It consists of 14 covalent bonds and two

[¶]We have assumed that the reduction potential of RSSR/RSSR⁻ in the blue and purple azurins is similar to that of 5,5'-dithiobis(2-nitrobenzoic acid) modified hemoglobin (47). Under this assumption, intramolecular ET in both wild-type blue copper and purple Cu_A azurin should, according to Eq. 4, exhibit a near-zero activation enthalpy (because $-\Delta G^0 \approx \lambda$). The relatively high activation enthalpy observed (see above) must mean that $-\Delta G^0 \neq \lambda$, and one possibility is that the potential of the RSSR/RSSR⁻ couple is more negative in the azurins. Our comparison of ET properties of blue and purple copper centers should be valid, however, because the same RSSR⁻ center serves as the electron donor in both systems.

hydrogen bonds. The coupling via this pathway would be much more efficient than the one in the purple azurin (19 covalent bonds, two hydrogen bonds, and one through-space-jump), which may explain the faster ET rate.

Theoretical calculations performed by Larsson *et al.* (27) and Gamelin *et al.* (11) have supported the idea that the advantage of the binuclear structure of the Cu_A center is in lowering both the inner and outer sphere reorganization energy compared with the T1 copper site. Their findings have been corroborated by the x-ray absorption study of Blackburn *et al.* (14), who observed minimal structural changes of the mixed valence binuclear center upon reduction.

In conclusion, by converting the single blue copper center into a binuclear Cu_A site and studying their ET reactivities in the same protein framework, we have demonstrated that the purple Cu_A centers are more efficient ET mediators than the blue copper center, mainly because of the low reorganization energy of the mixed-valence [Cu(1.5)-Cu(1.5)] site.

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